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TITLE CD-TOLERANCE IN PLANT CELLS: A COMPARISON OF BIOCHEMICAL AND MOLECULAR PROPERTIES OF TOLERANT AND SENSITIVE CELLS

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Cd-TOLERANCE IN PLANT CELLS: A COMPARISON OF BIOCHEMICAL AND MOLECULAR PROPERTIES OF TOLERANT AND SENSITIVE CELLS[1]

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ABSTRACT Plants and plant cells can be selected for the ability to grow in the presence of normally toxic concentrations of certain trace metal ions. Metal-tolerance is often associated with the ability to produce large amounts of certain metal-binding polypeptides, poly(γ-glutamylcysteinyl)glycines. The ability to produce these polypeptides plays an important role in Cd-tolerance since inhibition of their synthesis results in rapid cell death in the presence of metal ions. However, Cd-sensitive cells are also capable of synthesizing equivalent amounts of these compounds. Therefore, some other biochemical or physiological mechanism must also contribute to tolerance. Molecular and biochemical properties of Cd-tolerant Datura innoxia cells grown in the presence and absence of Cd were compared to those of Cd-sensitive cells grown under the same conditions. Certain biochemical and molecular differences which may contribute to tolerance were apparent.

INTRODUCTION

Angiosperms have rapidly colonized environments containing high concentrations of certain metal ions (1).

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While much is known about the genetics and evolution of metal tolerance in plants, the physiological and biochemical basis of tolerance is not as well understood. Poly-(γ-glutamylcysteinyl)glycines [$(\gamma EC)_n G$, n=2-5] have been implicated in metal-detoxification in a number of plant species (2,3,4). These compounds are not directly encoded by structural genes but are products of a biosynthetic pathway which consumes glutathione (5). They are clearly involved in metal-tolerance, since inhibition of their synthesis results in rapid cell death (6). However, Cd-sensitive cells are also able to synthesize similar amounts of these compounds in the presence of Cd. This suggests that some other factors must also contribute to tolerance. This paper describes the results of experiments designed to determine differences between Cd-tolerant and sensitive cells derived from a common cell line.

METHODS

Maintenance of Plant Suspension Cultures and Measurement of Growth Rates.

Suspension cultures of Cd-tolerant and sensitive D. innoxia were maintained as described (7). Packed cell volumes of 5 ml of culture were used to determine growth rates of cultures (8).

Radioisotope Labeling of Cells.

Carrier-free [^{109}Cd]Cl_2, L-[^{35}S]cysteine (>300 Ci mmol^{-1}), L-[4,5-^{3}H(N)]leucine (50 Ci mmol^{-1}) and [6-^{3}H]uridine (25-30 Ci mmol^{-1}) were purchased from E.I. du Pont de Nemours & Co., Inc., Boston, MA. Labeling was as described previously (2,5,7).

Determination of RNA and Protein Synthesis.

Cells were pulse-labeled with either 10 μCi/1.0 μg/ml [^{3}H]leucine or 5 μCi/0.1 μg/ml [^{3}H]uridine for 30 minutes. To determine uridine incorporation, four 5 ml aliquots were centrifuged at 800 g for 2 minutes to collect the cells. Pellets were then resuspended in 5 ml ice-cold 20% trichloroacetic acid (TCA) for 30 minutes. Cells were collected by centrifugation as before and the pellet was washed three times with ice-cold 5% TCA. Cells were then collected on glass fiber filters by vacuum filtration. Filters were washed five times with 5 ml ice-cold 5% TCA and twice with

95% ethanol, dried and assayed by scintillation spectrophotometry. Leucine incorporation was measured as described previously (5).

Cell Extraction and Analysis of Polypeptides.

Cells (10 ml of culture) were collected by centrifugation at 800 g for 1 minute, resuspended and washed twice in ice-cold buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM KCl, and 1.5 mM $MgCl_2$. Pellets were resuspended in 0.7 ml washing buffer containing 50 mM 2-mercaptoethanol and were homogenized in an Elvehjem tissue grinder. Samples were centrifuged (20,000 g for 30 minutes) prior to analysis by gel filtration HPLC. Extracts to be analyzed by reverse phase HPLC were acidified by addition of 0.6 ml of 1 N HCl then centrifuged for 10 minutes at 10,000 rpm in a microcentrifuge prior to passage through Centricon filters (Amicon Corp., >30,000 d exclusion) and analysis.

In vitro Translation.

Total RNA and (A^+)RNA were purified as described previously (5). (A^+)RNA was translated in a reticulocyte lysate kit (Amersham Corp.) using [^{35}S]methionine as the labeled amino acid. The procedures that accompanied the kit were followed for the translation of 1 μg (A^+)RNA. Translation products were separated by 2-dimensional gel electrophoresis using the method of O'Farrell (9) with the following modification; CHAPS was used instead of NP-40 in the first dimension and the first dimension rod was not equilibrated prior to electrophoresis through a 7.5-15% SDS gradient gel. Gels were impregnated with ENHANCE (E.I. du Pont de Nemours & Co., Inc.) prior to fluorography at -70°C.

HPLC Analysis.

Reverse phase HPLC. Two hundred μl aliquots of acidified cell extracts were applied to a 250 x 4.6 mm column of nucleosil C-18 (BioRad) equipped with a 30 x 4.6 mm precolumn. Samples were eluted with a 20 ml linear gradient of 0.1% (v/v) trifluoroacetic acid to 0.1% trifluoroacetic acid containing 20% (v/v) acetonitrile, at a flow rate of 2 ml min^{-1}. One ml fractions were collected and assayed for the presence of thiols.

Gel filtration HPLC. Twenty μl aliquots of cell extracts were applied to two 300 x 7.5 mm columns (in series) of Spherogel-TSK 3000SW (Beckmann Instruments) equilibrated

with 50 mM Tris-HCl, pH 7.0, 150 mM NaCl. Samples were eluted with the same buffer (0.5 ml min^{-1}) and 250 μl fractions were collected and assayed for the presence of ^{109}Cd.

Thiol Assay.

The total number of thiol groups was determined by the method of Ellman (10).

RESULTS

Growth of cells in the presence or absence of Cd. Cultures of Cd-sensitive Datura innoxia cells and cells tolerant of long-term exposure to 250 μM Cd divide with similar frequencies in the absence of the toxic metal ion. Exposure to 250 μM Cd results in an initial decrease in the viability of both cultures as determined by the exclusion of trypan blue (Fig. 1). However, while the viability of the tolerant culture stabilizes at approximately 80% and eventually recovers, the viability of the sensitive culture is reduced to approximately 20% within 48 hours after exposure to Cd and continues to decline.

Protein and RNA synthesis. Cd-tolerant and sensitive cells were exposed to 250 μM $CdCl_2$ for different periods. Cells were then incubated for 30 minutes in either [^{3}H]-leucine or [^{3}H]uridine to determine the rate of protein and RNA synthesis occurring in the cells. Figure 2 shows a rapid decrease in RNA synthesis in sensitive cells within eight hours after exposure to Cd. This is followed by a rapid decrease in protein synthesis. Both RNA and protein synthesis decrease slightly in Cd-tolerant cells but recover to previous levels.

Synthesis and assembly of Cd-binding complexes. Initially, Cd-tolerant and sensitive cells synthesize equivalent amounts of $(\gamma EC)_n G$ upon exposure to 250 μM Cd (Table 1). The amount of synthesis is similar for 24 hours. After this time there is a decrease in the amount of these compounds accumulated in the sensitive cells. However, the viability of the sensitive culture has decreased by this time. This suggests that the ability to synthesize metal-binding polypeptides is only part of the tolerance mechanism. Analysis of metal-binding complexes in the two cell cultures demonstrates a difference in the amount of Cd bound shortly after exposure of the cells to Cd (Figure 3). In the first 4 hours (Fig 3a), most of the Cd found in extracts from the sensitive cells elutes at a Kd similar to a

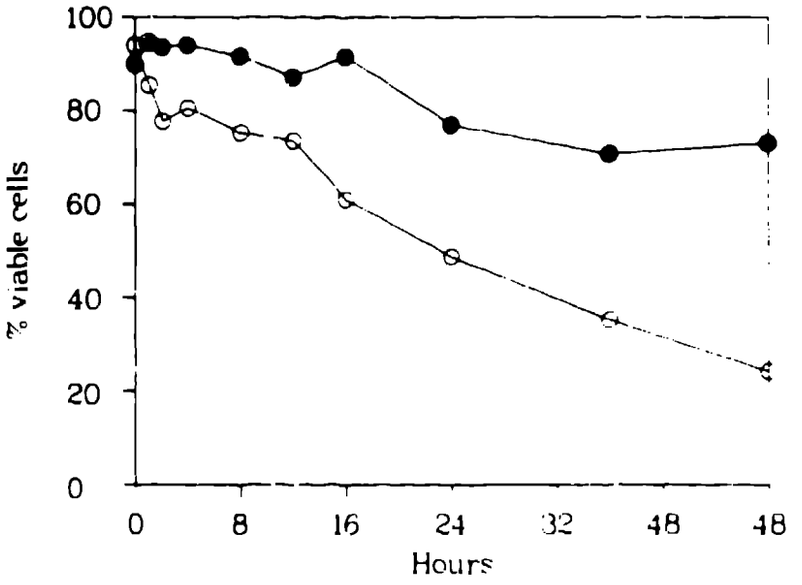

FIGURE 1. Determination of cell culture viability by exclusion of a vital stain. A small sample of culture was placed on a microscope slide and stained with a solution containing 2% (w/v) trypan blue. After five minutes cells were viewed microscopically and at least 800 cells were scored for the absence or presence of the dye within the cytoplasm. (●), Cd-tolerant cells; (o), sensitive cells.

2-mercaptoethanol:Cd complex (fraction 41) while a portion of the Cd found in extracts from Cd-tolerant cells is associated with metal complexes known to contain $(\gamma EC)_nG$. After 8 hours (Fig. 3b), the majority of the Cd in extracts from tolerant cells is associated with these complexes. After 48 hours (Fig. 3c), sensitive cells have formed larger Cd:$(\gamma EC)_nG$ complexes but, by this time, cell death has occurred.

Analysis of in vitro translation products by two-dimensional gel electrophoresis. Total mRNA was isolated from Cd-tolerant and sensitive cells grown in the absence of Cd and from cells exposed to 250 μM Cd for four hours. Figure 4 shows the results of 2-dimensional gel electrophoresis of the products of in vitro translation of this mRNA. Results demonstrate that some mRNA sequences are synthesized constitutively by tolerant but not by sen-

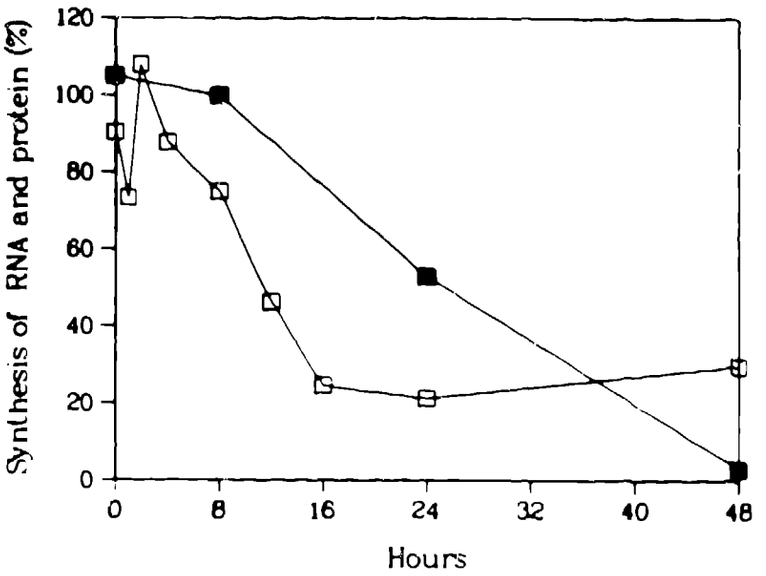

FIGURE 2. RNA and protein synthesis in Cd-tolerant and sensitive cells exposed to Cd for different periods. Cd-tolerant and sensitive D. innoxia cells growing in the presence or absence of Cd for different periods were pulse-labeled for 30 minutes with either [^{3}H]leucine or [^{3}H]-uridine. Total radioactivity incorporated during this time was then determined. The rates of RNA (□) and protein (■) synthesis for sensitive cells are shown as a percentage of the corresponding rates for tolerant cells.

sitive cells in the absence and presence of Cd (panels a and b, arrows for example). In addition, some mRNA sequences increase in the presence of Cd in both tolerant and sensitive cells (panels b and d, arrows for example) while others decrease. The activities of several genes are therefore modulated, either directly or indirectly, by exposure of the cells to Cd.

DISCUSSION

The ability to synthesize large amounts of $(\gamma EC)_n G$ has been correlated with the ability of plants and plant cells to grow in the presence of normally toxic concentrations of certain trace metal ions (2,3,4,6,7). These compounds play an important role in metal-tolerance since the inhibition of their synthesis results in rapid cell death (6 and our

TABLE 1.
SYNTHESIS OF $(\gamma EC)_n G$ IN Cd-TOLERANT AND SENSITIVE D. INNOXIA CELLS AFTER EXPOSURE TO Cd*.

	μg GSH equivalents/mg protein	
	Cd-tolerant cells	Cd-sensitive cells
Hours		
0	0	0
8	0.17	0.23
24	0.77	0.86
48	1.32	0.52

*Extracts from cells were separated by reverse phase HPLC and the amounts of thiols present in each fraction determined. Fractions containing material with retention times equal to the different $(\gamma EC)_n G$ were used to determine the total number of thiols, expressed as μM GSH equivalents/mg protein, and, therefore, the total amount of these polypeptides present. Time is the number of hours after addition of Cd.

own unpublished results). The enzyme(s) responsible for the synthesis of these compounds are present constitutively in both Cd-tolerant and sensitive cells, even in the absence of toxic metal ions (5). This suggests that either the metal-binding polypeptides or the enzymes perform some other metabolic function in the absence of metal ions. While $(\gamma EC)_n G$'s play a role in metal tolerance, the mechanism of resistance is more complex. Both Cd-tolerant and sensitive cells can synthesize these compounds upon exposure to Cd. However, only the tolerant cells survive. There are clearly other factors which contribute to tolerance.

Results presented here demonstrate a clear difference in viability between Cd-tolerant and sensitive cells exposed to Cd. This is first manifested in sensitive cells as a reduction in rates of RNA and protein synthesis and is followed by a rapid reduction in viability of the culture. However, these events precede any effects of Cd on $(\gamma EC)_n G$ accumulation in sensitive cells. Therefore, some other factor(s) must be involved in tolerance. Equivalent synthesis of $(\gamma EC)_n G$ is not accompanied by equivalent accumu-

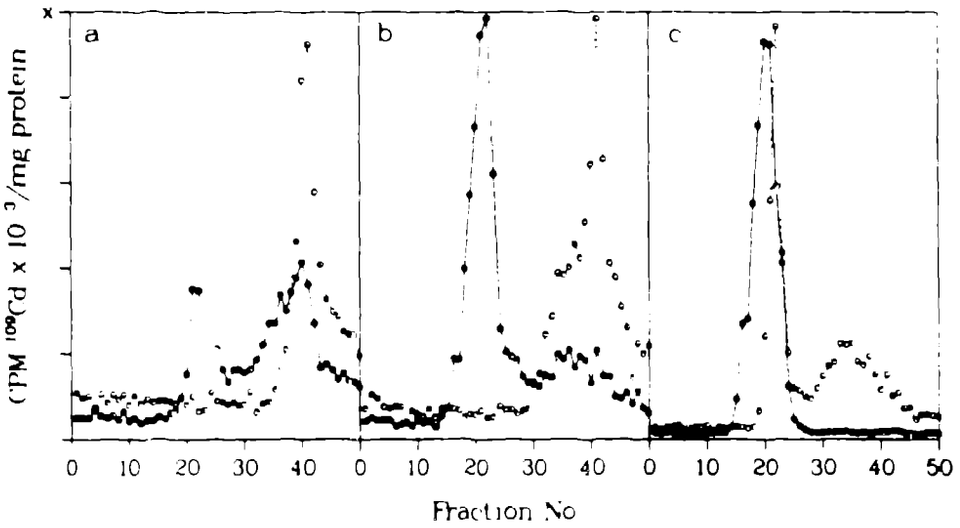

FIGURE 3. The accumulation of Cd-binding complexes in Cd-tolerant and sensitive cells exposed to $CdCl_2$ for different periods. Cd-tolerant and sensitive D. innoxia cells were grown in media containing 250 μM $CdCl_2$ for 4, 8, and 24 hours (panels a, b, and c, x = 50, 70, and 325 respectively). They were then extracted and analyzed by gel filtration HPLC. (●), Cd-tolerant cells; (o), sensitive cells.

lation of Cd^{2+} in metal-polypeptide complexes in the first four to eight hours after exposure to Cd. In some mammalian cells, tolerance to different concentrations of Cd is tightly correlated with the ability to produce specific amounts of metallothionein immediately following exposure to Cd (11). The ability to form Cd:$(\gamma EC)_n$G complexes during initial exposure to Cd may determine whether or not cells can survive long-term exposure to this metal ion. The results presented here suggest that Cd-sensitive cells are unable to form $(\gamma EC)_n$G:Cd complexes during early periods of Cd exposure. This may result because the polypeptides are somehow separated from the Cd in the cells. Alternatively, the size or structure of the polypeptides may differ between the tolerant and sensitive cells. Reese and Winge have reported that sulfide is a component of polypeptide:Cd complexes isolated from Schizosaccharomyces pombe (12). It is possible that tolerant cells are able to sym-

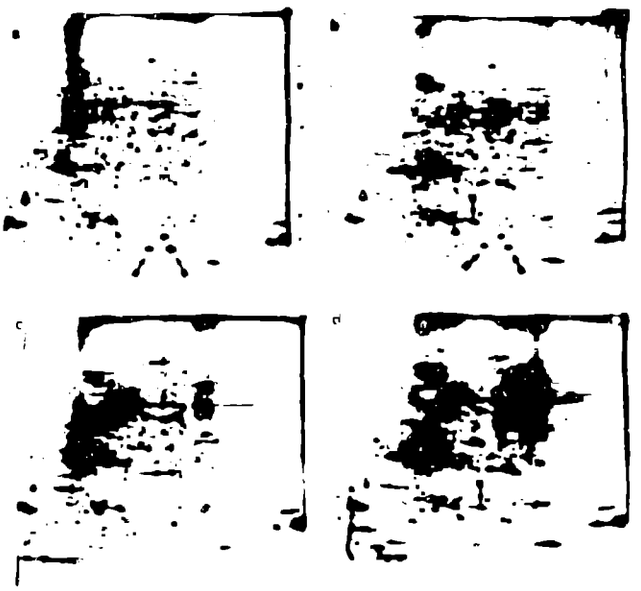

FIGURE 4. Two-dimensional gel electrophyoresis of in vitro translation products of mRNA isolated from (a) Cd-tolerant cells growing in the absence of Cd; (b) Cd-tolerant cells growing for ' hours in 250 μM $CdCl_2$; (c) Cd-sensitive cells growing in the absence of Cd; and (d) Cd-sensitive cells growing for 4 hours in 250 μM $CdCl_2$. Arrows common to panels a and b represent products encoded by mRNA sequences which are constitutively expressed in Cd-tolerant cells growing in the presence and absence of Cd. Arrows common to panels b and d represent products encoded by mRNA sequences which are induced by Cd.

thesize sufficient sulfide during early exposure to Cd to form stable Cd complexes while sensitive cells lack this ability.

An analysis of the products of in vitro translations of mRNA demonstrates several differences in gene expression between tolerant and sensitive cells. Several genes which are not expressed in sensitive cells are constitutively expressed in their tolerant counterparts in the presence and absence of Cd and might encode "tolerance" proteins. The mRNAs induced by Cd in both cell lines might encode enzymes of the $(\gamma EC)_n G$ biosynthetic pathway. Although the initial synthesis of $(\gamma EC)_n G$ is cycloheximide insensitive (5), continued synthesis may require induction of the synthesis of these enzymes.

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